Calpain Inhibitor AK295 Protects Neurons From Focal Brain Ischemia

Effects of Postocclusion Intra-arterial Administration

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Background and Purpose This research was performed to determine whether a selective inhibitor of the calcium-dependent protease, calpain, could reduce ischemia-associated brain damage when peripherally administered after a vascular occlusion.

Methods A variation of the rat middle cerebral artery occlusion model was used. A range of doses of AK295 (a novel calpain inhibitor synthesized for this purpose) was continuously infused through the internal carotid artery, beginning 1.25 hours from the initiation of the occlusion. Rats were killed at 21 hours, and the infarct volume was quantified.

Results Postocclusion (1.25-hour) infusion of the calpain inhibitor AK295 elicited a dose-dependent neuroprotective effect after focal ischemia. The highest dose tested (3 mg/kg per hour) afforded the maximum effect, illustrated by a 32% reduction in infarct volume 21 hours after the ischemia (vehicle, 81.7±4.7 mm^3; AK295, 54.9±6.9 mm^3; $P<.007$).

Conclusions These data provide the first evidence that a peripherally administered calpain inhibitor can protect against ischemic brain damage. They offer further support for an important role of calpain proteolysis in the brain degeneration associated with cerebral ischemic events and suggest that selective calpain inhibitors provide a rational, novel, and viable means of treating such neurodegenerative problems. (***Stroke***, 1994;25:2265-2270.)

Key Words • calcium • calpain • cerebral ischemia • rats • neuroprotection

Stroke remains a major source of mortality and disability in society. In recent years our understanding of the metabolic processes that render neurons susceptible to ischemic injury has improved. In this context, calcium flux has assumed a pivotal role as a key event that precipitates neuron death, and for this reason various pharmaceutical approaches have been used to control elevations in intracellular calcium concentrations.[1-4] However, it remains unclear precisely why increases in intracellular calcium lead to cell death. Clearer insight into the specific intracellular calcium-dependent events that are most cytotoxic to neurons would benefit the development of more effective pharmaceuticals.

One event that has generated recent interest is ischemia-induced activation of the cytosolic proteases, calpains. Calpains are a family of homologous, neutral thiol enzymes that normally exist in a quiescent state. Calcium-dependent activation of calpain occurs by autolysis of a portion of the N terminus of both the catalytic and regulatory subunits, presumably exposing the active proteolytic site. (For a series of reviews, see Reference 5.)

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The number and breadth of cellular proteins that serve as substrates for calpain are manifold.[5-11] Thus, prolonged activation of calpain will have a variety of detrimental effects on neurons, including compromising the integrity of the phospholipid membrane of the cell, interfering with exclusion of calcium through receptor and/or ion channel complexes, compromising transport of essential cell products to and from the cell body and terminal areas, and impairing numerous signaling events within the cell. When unregulated, any one of these events would be expected to compromise the function and vitality of the cell. When several such events are induced simultaneously, their cumulative consequences will most certainly be lethal.

Recent studies in animal models have supported the deleterious role of calpain in the neurodegenerative pathology of cerebral ischemia. For example, it has been demonstrated that significant calpain activation occurs before the apparent cell death that follows global[10] and focal ischemia.[11] Additionally, when the general serine/thiol protease inhibitor leupeptin was infused intraventricularly for 3 days before global ischemia in the gerbil, significant neural protection and inhibition of spectrin proteolysis was observed, suggesting that calpain inhibition might be responsible for both effects.[10] Furthermore, when the calpain inhibitor E-64c was administered to rats subjected to focal ischemia both before and after the ischemic insult, significant reduction in proteolysis of one calpain substrate (MAP2) but not of another (myelin-associated glyco-

protein) was observed; unfortunately, no histological or other means of assessing cell viability was reported.[12] Most recently, more direct evidence for a prominent role of calpain proteolysis and potential for calpain inhibitors in rats was reported. It was demonstrated that direct supracortical application of a potent and relatively selective calpain inhibitor, AK275, afforded significant dose-related neuroprotection in a rat model of focal ischemia. Moreover, the robust protection could be achieved even when application of the inhibitor was initiated 3 hours after the onset of the occlusion.[13]

Thus, the available evidence is collectively consistent with the idea that unregulated proteolysis by calpain represents a final common pathway to cell death. As such, drugs that selectively inhibit calpain may provide a novel and potentially powerful means of reducing the damage normally associated with stroke and related brain ischemia.[14] Taken together, these data provide the empirical foundation for the emerging "calpain hypothesis of ischemic brain damage." Clinical application of the calpain hypothesis will be determined in part by the ability of calpain inhibitors to protect against neurodegeneration when administered peripherally at time points after the initiation of the ischemic event.

The present study provides the first evidence of significant reduction in infarct volume with peripheral infusion of a calpain inhibitor after ischemic insult.

Materials and Methods

Subjects and Vivarium Conditions

One hundred nineteen Sprague-Dawley rats (250 to 350 g, Taconic) were used for these studies. The animals were acclimated to our vivarium for 5 to 14 days before surgery and were housed in polycarbonate cages filled with hardwood bedding. Water and food (Purina Laboratory Rat Chow, #5001, Purina Mills) were available ad libitum throughout the entire experiment. The vivarium was maintained at 22±1°C, with a relative humidity of 40±4%, on a 12-hour light/dark schedule.

Rat Model of Focal Cerebral Ischemia

Rats were subjected to distal occlusions of the parietal branch of the left middle cerebral artery (MCA), essentially in accordance with Chen et al,[15] as described elsewhere.[13] Briefly, all rats were anesthetized intramuscularly with a 4-mL/kg mixture of ketamine (25 mg/mL), xylazine (1.3 mg/mL), and acepromazine (0.33 mg/mL) after premedication with atropine methyl bromide (0.1 mg/kg IP). The common carotid arteries (CCAs) were isolated through a ventral midline cervical incision. All animals then underwent a subtemporal craniectomy to expose the left MCA.

The parietal branch of the MCA was permanently occluded up to 2.0 mm distal from the frontal/parietal bifurcation, using a single 10-0 suture (Ethicon Inc) and bipolar coagulation and taking care to include within the occlusion any large arterial branches. Immediately after MCA occlusion (MCAO), the CCAs were transiently occluded (1 hour) using atraumatic aneurysm clips (Roboz Surgical Instruments), and the opening in the skin was closed with wound clips.

The branching pattern of the MCA was drawn and recorded for each rat before occlusion and at the time of death. Ten percent of animals were removed from the study at the time the MCA was exposed (before MCAO) because of multiple, major branching. An additional 5% of rats were removed at time of death before staining with 2,3,5-triphenyltetrazolium chloride (TTC) because of the presence of a cortical branch originating below the level of the cranial window and supplying blood to the parietal cortex.

TABLE 1. Mean Temperature of Rats During and After MCAO Procedure

	Vehicle, °C	1.5 mg/kg, °C	3.0 mg/kg, °C
Pre-op	37.0±0.6	37.2±0.5	37.1±0.7
Occlusion	37.5±0.6	37.6±0.5	37.0±0.8
Reperfusion	37.4±0.4	37.8±0.4	37.4±0.3

Body temperature (mean±SD, rectal probe) from rats treated with vehicle and the two highest doses of AK295. Temperature was continuously monitored and controlled, with values recorded from each animal immediately before surgery (pre-op), at the time of middle cerebral artery occlusion (MCAO), and on release of the carotid clips after 1 hour of bilateral common carotid occlusion (reperfusion). No differences in temperature were observed between time points within the MCAO procedure or among treatment conditions. (See text for description of conditions.)

Body temperature was continuously monitored (via rectal probe), recorded, and maintained normothermic throughout and after the MCAO procedure by placing the rats on heating blankets connected to a rectal thermistor probe (Harvard Apparatus). Very little variation (and no consistent group differences) in body temperature was observed in any of the present studies (Table 1). The body temperature of any individual animal never varied by more than 1°C.

During the 1-hour CCA occlusion, a catheter was implanted into the external carotid artery (ECA) to enable intra-arterial administration of the calpain inhibitor through the internal carotid artery (ICA). One hour after the induction of the MCAO, the aneurysm clips were removed to permit reperfusion of the CCAs, and the wound was closed. Rats were then placed in a cage for monitoring and drug administration (see "Drug Administration" below). All animals were killed at 21 hours after MCAO, as described below. At the time of death, rats subjected to MCAO did not exhibit unusual psychomotor or other overt behavioral abnormalities (eg, sedation, rotation, ambulatory changes, etc) unrelated to the general surgical intervention: no apparent behavioral consequences of the brain infarct were observed.

Tissue Processing

Twenty-one hours after the induction of focal ischemia, rats were deeply anesthetized with CO_2 and decapitated, and their brains were rapidly removed. The cerebrum was chilled in ice-cold 0.9% saline for 10 minutes (0°C to 4°C) using a chilled brain matrix (Model REM4000C, Activational Systems Inc). Seven 2.0-mm coronal sections were cut from the cerebellum forward, immersed in 10 mL of a 2.0% solution of TTC (Sigma Chemical Co),[16,17] and placed in a 37°C incubator for 30 minutes (each surface facing up for 15 minutes).

After staining, sections were washed twice in 10 mL 0.9% saline, placed in 10 mL 10% formalin solution, and left at 4°C overnight in a lightproof container. After two additional washes in 0.9% saline (10 mL), computer digitization of each section and subsequent image analysis were undertaken.

Tissue Section Analysis

The subsequent digitizing, computer imaging, and infarct quantitation were performed under blinded (ie, coded) conditions. Digitized enlarged (×16) images of the caudal and rostral sides from each section (a total of 14 images per brain) were generated and stored using Macintosh PHOTOFLASH 1.0 software (Apple) and a Kodak professional DCS200 digital camera. The areas of interest were highlighted and analyzed using the image processing and analysis program, IMAGE v1.44 (National Institutes of Health public-domain software). The infarcted area (white tissue) and "total damage" area (infarcted plus pink-stained tissue) were then computed from these

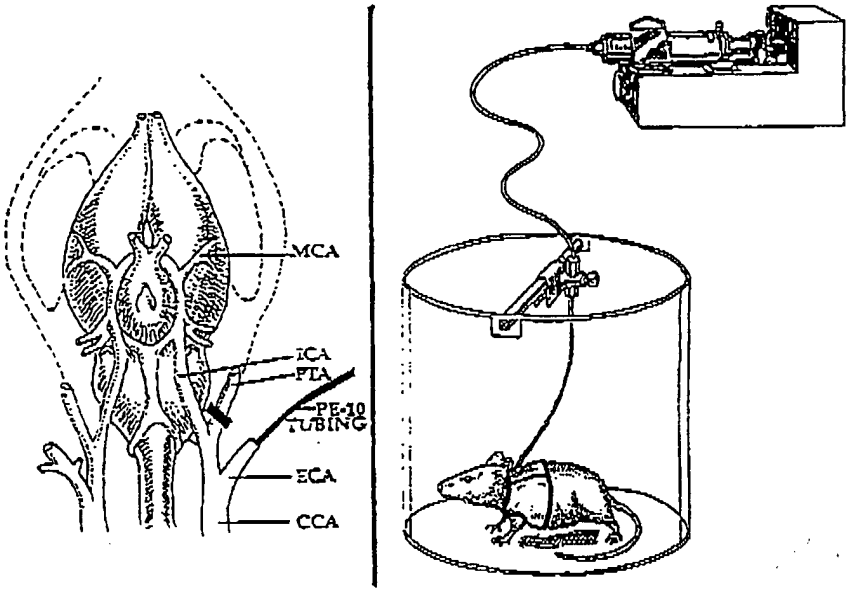

FIG 1. Diagram of system that permitted the continuous intra-arterial infusion of drug into awake, freely moving rats. Left, The drug is infused retrogradely into the external carotid artery (ECA) ipsilateral to the occluded middle cerebral artery (MCA). With the pterygopalatine artery (PTA) cauterized, the drug flows from the ECA through the internal carotid artery (ICA) and into the circle of Willis. CCA indicates common carotid artery. Right, See text for detailed explanation of setup. The major components are the syringe pump, fluid swivel, jacket with spring tether, and the observation holding cage.

digitized images. We have found that infarcts quantified in this fashion correlate very well ($r > .99$) with infarcts from the same tissue quantified by immersion-fixed hematoxylin and eosin staining (results not shown).

The total and infarct volumes of cortical ischemic tissue induced by the MCAO were calculated for each animal by measuring the area of damage per section and converting that value to volume per brain using a standard trapezoid equation.[13]

Characteristics of AK295

AK295 (Cbz-Leu-aminobutyrate-CONH$(CH_2)_3$morpholine) derives from a family of dipeptide α-ketoamide calpain inhibitors that were synthesized and developed as potential drugs for treating ischemic brain damage.[18,19] AK295 was specifically synthesized to be more soluble in aqueous media than are other family members, such as AK275 (the compound used for the supracortical-application study discussed earlier).[13] Thus, AK295 was used in this study because it allowed considerably higher concentrations of drug to be administered while still preserving the requisite profile of high potency, selectivity, and neuronal permeability shared by other members of the family (in preparation).

Drug Administration

After induction of focal ischemia, a 40-cm length of PE-10 tubing containing heparinized saline (50 U/mL) was exteriorized at the back of the neck, and the free end was left at the surgical site. The left ICA and ECA were exposed at the carotid bifurcation. In addition, the left pterygopalatine artery was occluded by bipolar coagulation. The ECA was ligated with a 5-0 silk thread approximately 4 mm from the carotid bifurcation. The free end of the PE-10 tubing (50 cm) was inserted approximately 3 mm into the ECA, up to the bifurcation, and secured in place using 5-0 silk thread and tissue adhesive (Vetbond, 3M). The tubing was threaded through a spring tether that was attached to the rats by an external harness worn over their shoulders (Rodent Infusion Set, KLASS). The free end of the PE-10 tubing was interfaced to the outflow of a fluid swivel with a 2-cm piece of PE-50 tubing. The inflow end of the swivel was connected to a 55-cm length of heparinized PE-50 tubing that, in turn, was connected to a continuous-drive syringe pump (Razel). Animals were housed individually in plastic 5-gallon buckets during drug infusion and were given access to food and water ad libitum. This system permitted continuous intra-arterial infusion of vehicle or drug to a freely moving animal (Fig 1).

In the present study, a range of concentrations (0.1 to 2 mmol/L) of the calpain inhibitor AK295 were prepared in saline with an equimolar amount of acetic acid to produce the acetate salt (Cbz-Leu-Abu-CONH$(CH_2)_3$Mpl · AcOH). Each concentration was administered in two phases after occlusion. First, a "priming dose" of 1.5 mL administered over 10 minutes (9 mL/h) was infused beginning 75 minutes from the induction of focal ischemia. This was followed by a continuous, slower infusion (0.7 mL/h), which persisted until the rats were killed 21 hours after MCAO. Thus, the total volume each rat received was approximately 15.5 mL. The lowest concentration tested (0.1 mmol/L) corresponded to a dose of 0.15 mg/kg per hour, whereas the highest concentration (2 mmol/L) corresponded to a dose of 3.0 mg/kg per hour over a 20-hour treatment period. The minimal delay of 75 minutes used between induction of ischemia and initiation of drug treatment was selected as a practical consideration, in that the carotids were bilaterally occluded for 60 minutes and an additional 15 minutes provided a convenient period in which to connect the animal to the infusion system (Fig 1).

Results

These studies successfully established a dose-dependent neuroprotective effect of the calpain inhibitor AK295 in our rat model of focal ischemia (Table 2). As such, AK295 ameliorated the effects of MCAO by more than 30%, using both the infarct-only and total-damage measurements (Figs 2 and 3). Statistical tests using ANOVA confirmed significant protective effects of AK295 with both measures [total damage: $F(5,113)=3.22$, $P<.009$; infarct only: $F(5,113)=2.56$, $P<.03$].

Two-tailed t tests were used to make individual dose versus vehicle comparisons and indicated that both 1.5 and 3.0 mg/kg per hour elicited similar neuroprotection (total damage at 1.5 mg/kg per hour: $t=2.28$, $df=69$, $P=.01$; at 3.0 mg/kg per hour: $t=3.11$, $df=77$, $P=.001$; infarct only at 1.5 mg/kg per hour: $t=2.04$, $df=69$, $P=.02$; at 3.0 mg/kg per hour: $t=2.79$, $df=77$, $P=.003$). The dose of 0.75 mg/kg per hour of AK295 provided some protection, although this was less robust and consistent than that seen with the two highest doses (Table 2). The two lowest doses of AK295 used, 0.15

TABLE 2. Reductions in Ischemic Damage by AK295

AK295 Dose, mg/kg per h	n	Infarct Volume, mm³ (Mean±SEM)	Decrease, %	Total Volume, mm³ (Mean±SEM)	Decrease, %
Vehicle	61	81.1±4.7	...	106.7±5.3	...
0.15	9	73.7±13.9	9	116.0±16.4	−9
0.30	10	71.1±9.9	12	92.2±11.3	14
0.75	11	55.3±11.3*	32	80.4±14.1	25
1.5	10	56.1±10.0*	31	74.6±13.3*	30
3	18	54.9±6.9†	32	74.0±7.5†	31

Mean±SEM volumes for infarct only and total damage in vehicle- and drug-treated rats 21 hours after focal ischemic insult. Infarct indicates nonstained tissue completely lacking detectable mitochondrial activity; Total, tissue that is nonstained (ie, infarct) plus all poorly-stained, apparently impaired tissue.

*$P<.05$; †$P<.01$ with a two-tailed t test.

mg/kg per hour and 0.30 mg/kg per hour, were ineffective ($P>.10$).

Discussion

These data provide the first evidence that a peripherally administered inhibitor of calpain can reliably reduce the magnitude of brain damage after a neural perturbation and offer direct support for calpain inhibitors as a therapeutic approach against ischemic brain damage. Significant reduction in volume of infarct was achieved when intra-arterial administration of the inhibitor was initiated after focal ischemia; this offers encouragement that calpain inhibitors may prove to be clinically useful drugs for protecting the brain against the ischemic injury that normally occurs after stroke and other clinical neural ischemic conditions. These data thus support and extend the recent positive findings achieved with an analogue (AK275) from the same family of calpain inhibitors. In that study, a robust (50% to 75%), dose-related reduction in infarct volume was achieved when the drug was applied directly to the vulnerable brain tissue, even when administration was begun 3 hours after induction of the ischemic insult.[13]

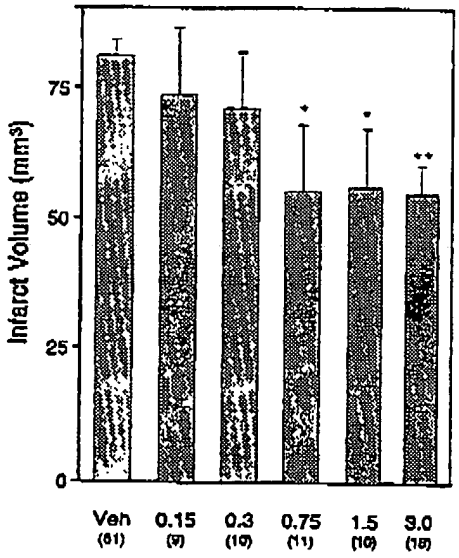

FIG 2. Bar graph shows effect of postischemic intra-arterial administration of the calpain inhibitor AK295. Doses refer to the infusion of drug in milligrams per kilograms per hour, initiated 1.25 hours after the MCAO and continuing for 20 hours, at which time the rats were killed. The small numbers in parentheses below each dose refer to the number of rats tested per treatment group. Data are represented as mean±SEM infarct volume (mm³). *$P<.05$ and **$P<.01$, compared with vehicle-treated rats, using two-tailed tests. (See text for more specific statistical information.)

Additional studies are required to confirm and extend the current effects achieved with peripheral administration. Determination of the maximal achievable therapeutic response (and dosing paradigm), the maximal duration of the therapeutic window, and the long-term persistence of the neuroprotective effects is key to evaluating the potential usefulness of calpain inhibition in treating neurodegeneration. Additionally, given the importance of calcium disregulation in many forms of neurodegeneration and the lethal consequences of uncontrolled calpain proteolysis, it would also be of value to determine which of the numerous types of acute (and perhaps chronic) brain degeneration might be most effectively treated by inhibition of calpain.

The present finding that peripheral postischemic administration of a selective calpain inhibitor can provide significant neuroprotection offers the best evidence to date that calpain inhibitors might ultimately be developed as effective drugs for neurodegenerative indications. There are numerous potential advantages of calpain inhibitors as neuroprotective drugs. Calpain activation is precipitated by elevations in intracellular calcium[5]; thus, activation of calpain and subsequent proteolysis represents a cytotoxic event that can be initiated by disregulation of any of the several glutamate receptors and ion channels thus far implicated in the ischemic pathogenic cascade. Calpain inhibitors therefore provide a point of therapeutic intervention that is common to the numerous receptor or channel targets that play important roles in calcium-mediated neurotoxicity.

A second related advantage of calpain as a therapeutic target is that, as an intracellular protease, its role is downstream from more conventional targets. Thus, while the release of glutamate, its binding to specific receptors, and the opening of ion channels are important (albeit not directly cytotoxic) events in the neurotoxic cascade, the activation of calpain occurs only after

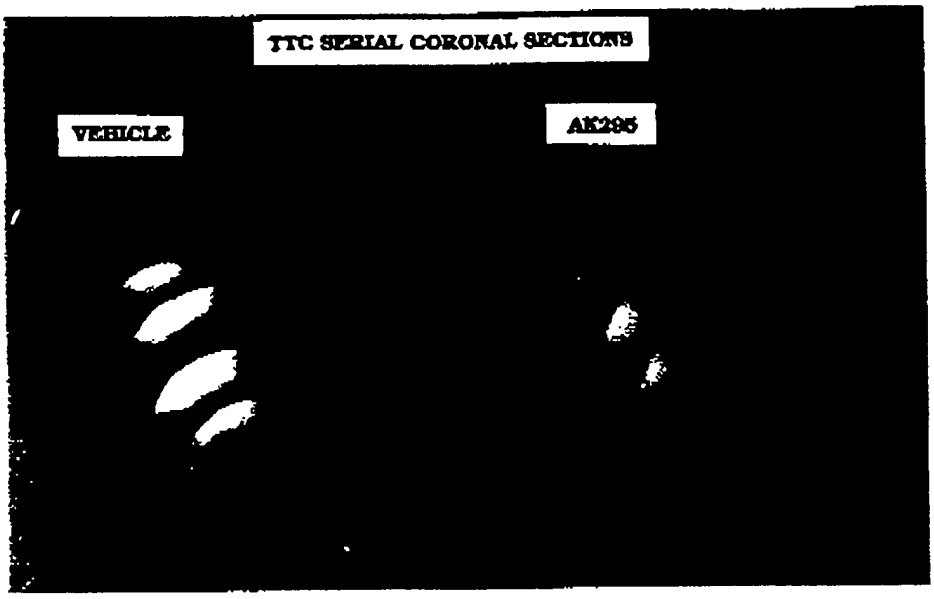

FIG 3. Representative serial 2,3,5-triphenyltetrazolium chloride (TTC)–stained brain sections illustrating the infarct volume of the vehicle-treated groups (mean infarct, 81.7 mm^3) or AK295-treated groups after postischemic intra-arterial infusion. Infusions of 0.75 to 30 mg/kg per hour of AK295 produced mean decreases in infarct volume of greater than 30% (administered 1.25 hours after occlusion), compared with the vehicle-treated group. Tissue was stained 21 hours after ischemia.

intracellular calcium reaches threshold levels. Thus, in addition to whatever time window for therapy exists with various extracellular approaches, an additional interval of time necessarily exists for calpain inhibitors. This temporal advantage arises because of the cumulative increased time required (1) for glutamate receptors to become adequately occupied and corresponding ion channels to open; (2) for calcium to reach effective activation levels; (3) for calpain, in turn, to become sufficiently activated; and (4) once activated, for calpain to progressively digest its substrates to the point where irreversible cell damage occurs. While the temporal limits of calpain inhibitors have yet to be defined (and in absolute terms will likely vary widely among different animal models and clinical conditions), recent findings with the MCAO model indicate that the window of opportunity for calpain inhibitors is at least several hours.[13] Thus, because calpain activation and substrate proteolysis represent a common late-stage event in the excitotoxic cascade that is likely to be directly cytotoxic, the therapeutic window for calpain inhibitors may be open wider and for a longer duration than the window for other therapeutic approaches.

Another potential advantage of calpain inhibitors is that, in addition to protecting the cell from the consequences of calcium influx, they offer the prospect of also protecting the cell from cytotoxic release of large intracellular calcium pools.[4] Unfortunately, therapeutic approaches aimed at extracellular targets such as receptors and channels cannot be expected to offer direct relief from this ischemia-induced, intracellular, potentially cytotoxic event.

Finally, it can be argued that calpain inhibitors should offer a therapeutic index that is much greater than that achieved with more conventional receptor- and ion channel–based approaches. This is because calpain normally exists in a quiescent or inactivated state, requiring the achievement of relatively high intracellular levels of calcium for activation of calpain to occur. Thus, activated calpain is unlikely to play a crucial role in normal moment-to-moment function and communication of neurons; the calcium levels required to activate calpain are rarely achieved and certainly not for extended durations. Given this, selective inhibitors of calpain would not be expected to have marked effects on normal brain function, a prediction thus far confirmed.[20-22]

In conclusion, the present data demonstrate that when a selective and membrane-permanent calpain inhibitor is peripherally administered after a stroke is induced, a significant reduction in brain damage is achieved. As the first evidence that calpain inhibitors can accomplish this when administered in this fashion, these data represent another advance in developing a novel and potentially powerful approach to treating brain injury. They therefore add to the collective findings supporting the "calpain hypothesis" of ischemic brain damage in that (1) calpain activation is an important pathogenic participant in ischemic brain injury; (2) blocking calpain proteolysis after an ischemic event has occurred can protect against impending neuronal damage; and (3) selective calpain inhibitors can be developed as drugs for treating the brain damage that normally accompanies strokes, head injury, cardiac arrest, and other forms of ischemia-related disorders. Current efforts using modified dosing paradigms as well as new derivatives of inhibitors will help determine the extent to which this last point proves true.

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Note Added in Proof. Following the review of this manuscript, a paper by Hong et al[23] appeared which also reports an effect of calpain inhibition on infarct size. The dipeptidyl aldehyde (Cbz-Val-Phe-H) at doses of

30 mg/kg and 60 mg/kg IV produced roughly an 18% decrease in infarct volume, relative to the mean control groups.

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Editorial Comment

Calpains are calcium-activated proteolytic enzymes. Activated calpains attack a wide variety of cellular proteins. These features render proteolysis by calpains a potential mechanism for cellular death in brain ischemia. In their article, Bartus et al report that inhibition of calpains with the synthetic calpain inhibitor AK295 reduced infarct size following arterial occlusion in the brain. These results provide strong pharmacological evidence that calpain-mediated proteolysis is an important step in neuronal death from cerebral ischemia. As the authors point out, since proteolysis is a relatively late event in the cascade that leads to neuronal death in ischemia, its inhibition may provide the opportunity for effective intervention a long time after the onset of ischemia. This may lengthen the therapeutic window.

In the experiments of Bartus et al, only one duration of ischemia was tested. It will be necessary to extend these experiments with different durations of ischemia to identify more precisely the therapeutic window for the administration of the calpain inhibitors. Also, it would be desirable to test the effect of AK295 in global ischemia.

AK295 is water soluble. One presumes, therefore, that it may not penetrate an intact blood-brain barrier. It is therefore possible that its effectiveness may be greater if it is administered after the ischemia has had time to break down the blood-brain barrier, thereby allowing penetration of the drug into the ischemic brain.

A final issue is whether calpain-induced proteolysis is the only mechanism by which increased intracellular calcium concentration leads to neuronal death or whether additional mechanisms exist. The effectiveness of inhibition of calpain in the experiments of Bartus et al suggests that calpain proteolysis is indeed a very important mechanism. However, additional experiments in other species, as well as with other models of ischemia, are necessary to establish this point.

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